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Note

Improved method for the isolation of RNA from (standing liquid cultures of) *Streptomycetes*

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Abstract

Streptomycetes are complex soil bacteria capable of producing aerial reproductive mycelium and secondary metabolites. We observed novel phenomena such as an extended life cycle including flotation and anaerobiosis using standing liquid cultures. This paper describes an improved method for isolating good quality RNA from standing liquid cultures of *S. coelicolor* via excellent cell lysis.

Keywords: RNA isolation; Streptomyces; Efficient lysis

Streptomycetes are mycelial soil bacteria that undergo a complex developmental cycle on solid media. Spores germinate and form a branched, vegetative mycelium. Several signals trigger the formation of aerial hyphae that differentiate further into reproductive chains of spores. Differentiation is accompanied by the production of secondary metabolites, e.g. antibiotics. Gene expression studies based on RNA isolation, e.g. Northern hybridization, S1 mapping, reverse transcription, have been crucial to provide insight into differentiation and antibiotic production (for reviews, see Hopwood, 1999; Chater and Horinouchi, 2003 and references therein).

Shaken liquid cultures of *S. coelicolor* do not differentiate. However, we showed recently that morphological differentiation of *S. coelicolor* also occurs in standing liquid minimal media involving an extended life cycle (Van Keulen et al., 2003). In addition, submerged hyphae in liquid minimal medium may attach to solid surfaces. Furthermore, liquid standing cultures rapidly become anoxic, implying the existence of metabolic pathways supporting anaerobic growth or enabling the organism to survive long periods of low oxygen conditions (Van Keulen et al., 2003). All these phenomena have not been studied so far although this growth condition is environmentally important. Standing liquid cultures may resemble

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flooded soils, a condition *S. coelicolor* may escape from by forming floating sporulating colonies.

In order to study expression of genes involved in differentiation and anaerobic metabolism in standing liquid cultures we used various RNA isolation methods described for Streptomycetes (Kieser et al., 2000; Kormanec and Farkašovský, 1994; La Farina et al., 1983; Veenendaal and Wösten, 1998) but we were unsuccessful in obtaining RNA of high quality and yield. With the methods available we were unable to harvest and lyse cells fast and efficiently which may have resulted in inductional stress responses and RNA degradation, and/or they require (expensive) chemicals that are not standard (Table 1).

This paper describes a new and fast procedure for the isolation of high quality RNA from *S. coelicolor* using a quick harvesting procedure followed by mycelium fragmentation facilitating superior lysis of cells and isolation of DNA-free RNA using a phenol/guanidine isothiocyanate containing solution and DNaseI treatment. This method was successfully applied to the isolation of RNA from standing liquid cultures of *S. coelicolor* (Fig. 1). The procedure used was the following. *S. coelicolor* spores were pregerminated, inoculated in liquid mNMMP (Van Keulen et al., 2003) at a density of 1×10^6 spores ml^{-1}, dispensed in 10×10 cm polystyrene dishes (Greiner) at 0.5 cm liquid height, and incubated at 30 °C. At day 5, when floating colonies start to produce chains of spores, total biomass (floating, submerged, and attached mycelium) from six dishes was harvested by scraping the bottom of the dishes with a razor blade followed by rapid filtration on ME24 membrane filters (0.2 μm, Schleicher and Schuell) using a vacuum pump. Mycelium was scraped from the membrane, transferred to a 1.5 ml vial, frozen in liquid nitrogen, and kept at − 80 °C. Efficient lysis without inducing a stress response

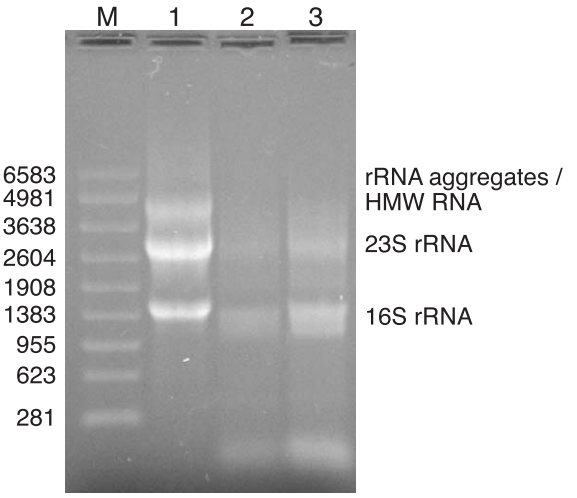

Fig. 1. Improved isolation of RNA of 5-day-old standing liquid cultures of *S. coelicolor*. M: RNA marker, numbers indicate bases. 5 μg RNA isolated with the method described here (lane 1), method 4 (lane 2), and method 6 (lane 3).

Table 1
Comparison of advantages and disadvantages of methods for RNA isolation from Streptomycetes

Method	Total time required	Advantages/disadvantages	Reference
(1) Kirby mix + phenol/chloroform + DNaseI	< 4 h	specialty chemicals required (e.g. triisopropylnaphtalene sulphonate, salicylate), lengthy	(Kieser et al., 2000)
(2) Kirby mix + CsCl gradient	1 h + ON centrifugation	as Kirby; long, expensive CsCl centrifugation	(Kieser et al., 2000)
(3) SDS + hot phenol	< 4 h	handling of hot solvents, lengthy	(Kieser et al., 2000)
(4) Kormanec and Farkasovsky	< 4 h	harvesting and lysis procedure invokes (cold) stress response, lengthy	(Kormanec and Farkašovský, 1994)
(5) La Farina et al.	< 2 1/2 h	harvesting and lysis procedure invokes (cold) stress response; handling of hot solvents	(La Farina et al., 1983)
(6) Veenendaal and Wösten	< 2 h	lysis by lysozyme invokes stress response	(Veenendaal and Wösten, 1998)
This method	< 2 h	harvesting and lysis procedure fast and under RNase inhibiting conditions; no time or condition for stress response; applicable to all types of cells; total time short	This study

was ensured by powdering the mycelium lumps first at − 196 °C using a mortar and pestle and liquid nitrogen. Mycelial powder was transferred to a new vial, 1 ml of a solution containing 50% (v:v) phenol and 30% (w:v) guanidine isothiocyanate solution (Trizol, Gibco) was added, mixed, and incubated at room temperature for 5 min. Subsequently, 0.2 ml of chloroform was added, carefully mixed, and left at room temperature for 2 min. The mixture was then centrifuged for 10 min and the upper phase was transferred to a new vial for DNaseI treatment using parts of a Wizard SV RNA isolation kit (Promega). Thus, 375 μl of SV RNA dilution buffer (Promega) was added to the nucleic acid solution, mixed by pipetting, and centrifuged for 10 min (blue pellet). Supernatant was mixed with 250 μl of cold 96% ethanol, loaded on a spin column (Promega), and centrifuged for 1 min. The column was washed with SV RNA wash solution (Promega) and incubated for 15 min with 50 μl of freshly prepared DNaseI incubation mix (Promega) at room temperature. The DNaseI reaction was stopped and the column was washed with SV RNA wash solution. RNA was eluted from the column by adding 50 μl of nuclease-free water. RNA concentration was determined on a Genequant spectrophotometer (Amersham Biosciences). To check the quality of the RNA, 5 μg of RNA was mixed with RNA denaturing solution, loaded on a denaturing formaldehyde agarose gel and electrophoresed for 1 h. For Northern hybridizations, RNA was blotted to a Nylon filter and hybridized at 63 °C under conditions described by Church and Gilbert (Church and Gilbert, 1984). 16S DNA probe was labelled with ^{32}P-dCTP using the Prime-a-Gene-Labelling kit (Promega).

Using this improved method, RNA was isolated at a higher yield (up to 200%) compared to other methods. The A_{260}/A_{280} ratio of the RNA preparation was between 1.9 and 2.0, illustrating good quality RNA with respect to purity. Fig. 1 shows that chromosomal DNA was absent. In addition, the 23S rRNA band was significantly more intense than the 16S rRNA band (Fig. 1, lane 1) indicating that RNA degradation did not occur. Bands between 23S and 16S and below the 16S band were present in lanes 2 and 3 but were absent in lane 1 which also indicated that RNA isolated with this method was not degraded. RNA present above the 23S rRNA band are high molecular weight (m)RNA molecules and rRNA aggregates. Total RNA from 5-day-old cultures isolated by this method and method 6 was hybridized with 16S DNA of *S. coelicolor* (Fig. 2). 16S DNA was hybridized to a single band in lane 1 representing 16 S RNA whereas lane 2 showed additonal signal below the intense band of 16S RNA indicating the presence of degraded RNA.

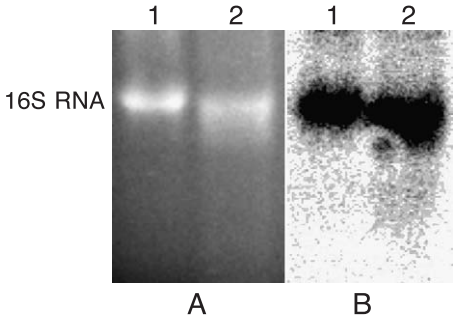

Fig. 2. Northern blot analysis of 16S rRNA. (A) Ethidium bromide-stained gel. (B) Northern blot of gel in A probed with ^{32}P-labelled 16S DNA. Lane 1, 5 μg RNA isolated with the method described here (lane 1), and method 6 (lane 2).

The method described here for the isolation of RNA from 5-day-old standing liquid cultures of *S. coelicolor* is clearly superior to conventional procedures. We were also able to isolate good quality RNA of *S. coelicolor* grown in standing liquid cultures throughout the extended life cycle of development (day 1 to day 8) or to stationary phase in solid and shaken liquid cultures (data not shown). RNA isolated via other methods was often degraded compared to RNA isolated with the method described here (Fig. 1).

In summary, the isolation of RNA using this method is fast and highly efficient. Also, its success has been confirmed with cells grown on solid media and in shaken liquid cultures of Streptomycetes (data not shown), with lactic acid bacteria, such as *Lactobacillus reuteri*, which are difficult to lyse, and with filamentous fungi (Resp. S. Kralj and Dr. A. Alves, personal communication). The method thus appears to be applicable for the isolation of RNA from a wide range of microorganisms.

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